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ORIGINAL ARTICLE

Application of response surface method to evaluate the cytotoxic potency of *Ulva fasciata* Delile, a marine macro alga

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Abstract Bioprospecting of marine natural products has recently produced a substantial number of drug candidates. *Ulva fasciata* Delile, belonging to the family Ulvaceae, is a green marine macro alga that grows profusely on the coastal seashore of South India. In the present study, we investigated the *in vitro* cytotoxic potential of a methanolic extract of *U. fasciata* Delile (MEUF) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against human colon carcinoma (HT-29), human hepatocyte carcinoma (Hep-G2), and human breast carcinoma (MCF-7) cell lines. Response surface methodology (RSM) was applied using central-composite experimental design (CCD) to obtain optimum combined effect of concentration and cancer cells with highest cytotoxicity. The effect of concentration, cancer cell lines as independent variables on absorbance (OD), percent cell survival and percent cell inhibition as dependent variables was investigated. Maximum cytotoxic activity of MEUF was established for Hep-G2 with lowest OD or percent cell survival; highest percent cell inhibition with significant difference ($p > 0.05$) was compared to HT-29 and MCF-7.

1. Introduction

The escalating requirements for drugs and serious adverse effects caused because of conventional synthetic medication enthused to seek new sources of natural products. Marine environment is becoming the most explored habitat because of its chemical and biological diversity. Among the marine sources, algae or seaweeds are the more valuable sources of

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structurally diverse bioactive compounds (Sullivan et al., 2010) which are proven as the key to combat various diseases. Even though, seaweeds are used as regular diet, the significance of algal food on human health is indistinct. Literature survey reveals that the beneficial effects of seaweeds and their bioactive substances like phlorotannins, sulfated polysaccharides, peptides and carotenoid pigments are diversified from eco-biotechnological to the industrial standpoint. Wijesekara et al. (2010) reviewed the biological activities of phlorotannins with potential health beneficial applications in functional foods, pharmaceuticals, and cosmeceuticals. Sulfated polysaccharides have several applications (Barahona et al., 2011) and exhibit various bio-activities such as anticoagulant (Mestechkina and Shcherbukhin, 2010), antiviral (Damontea et al., 2004), antimalarial (Dalton et al., 1991), anti-herpes (Wen et al., 2003), antiinflammatory (Matsui et al., 2003), immunestimulating (Yim et al., 2005), pulmonary fibrosis (Du et al., 2010), anticancer (Matsuda et al., 2003; Chen et al., 2010; Berteau and Mulloy, 2003; Campo et al., 2009) etc.

Although 'seaweeds' are consumed by Asians because of its known importance, most of the westerns do not think them as a nutritional or a daily supplement in their food. It is because of the term 'weed', which generally represents the unwanted plants in any ecosystem. Kim (2012) hence termed them as "sea-vegetables" to bring a positive notion in human beings to think 'algae' or 'seaweed' as consumable vegetables from sea.

Ulva fasciata Delile, belongs to the division: Chlorophycota, class: Ulvophyceae; order: Ulvales, and family: Ulvaceae. These are generously growing green marine macro alga in the costal seashore of south India. They reported to have various phytoconstituents like guaiane sesquiterpene derivatives, labdane diterpenoid with anti-microbial (Chakraborty et al., 2010a, 2010b) and sesquiterpenoids with outstanding antioxidant activity (Chakraborty and Paulraj, 2010). The alga ascertained excellent antiviral (Garg et al., 1992) and immunomodulatory activities (Selvin et al., 2004).

The present study aims to evaluate the cytotoxic potential of the methanolic algal extract using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against human colon carcinoma (HT29), human hepatocyte carcinoma (HepG2), and human breast carcinoma (MCF7) cell lines. Response surface methodology was used to know the influence of concentration and cancer cell lines on cytotoxicity.

2. Materials and methodology

2.1. Plant material collection and preparation of extract

The alga *Ulva fasciata* Delile was collected from the inter-tidal rocky surface of Visakhapatnam coast (Tenneti Park), Andhra Pradesh, India, during the month of May–June 2011 and authenticated at the department of Botany, Andhra University, Visakhapatnam 530003, A.P. The sample was thoroughly washed with fresh water followed by distilled water to remove the salt, epiphytes and other extraneous matter. It was then shed dried at room temperature, chopped into small pieces; coarsely powdered (500 g) was macerated in methanol (95% v/v) for one week. The green algal extract was subsequently filtered off and concentrated under vacuum at 40 °C (Buchi, Switzerland) to get a dark-green residue (5.7 g). The extract was stored in a vacuum desiccator until further use.

2.2. Chemicals and reagents

Minimal essential medium, fetal bovine serum and trypsin were obtained from Gibco's (USA). Amphotericin-B and MTT were purchased from Sigma–Aldrich (Mumbai, India). Beyond this, a list of highly pure chemicals like glucose, L-glutamine, penicillin-G, streptomycin, phosphate-buffered saline (PBS), ethylenediamine tetra-acetic acid (EDTA), and dimethyl sulfoxide (DMSO) lysis buffer, were supplied by Himedia (Mumbai, India). Cell culture flasks and 96-well plates were procured from Corning Incorporated (Corning, NY, USA). All the rest chemicals were of high purity and reagent grade.

2.3. Cell culture

Human cancer cell lines used in this study were procured from the National Centre for Cell Science, Pune. All cells were grown in the Minimal essential medium (MEM, GIBCO) supplemented with 4.5 g/L glucose, 2 mM L-glutamine and 5% fetal bovine serum (FBS) (growth medium) at 37 °C in 5% CO_2 incubator.

2.4. MTT assay

The MTT assay developed by Mosmann (1983) was modified and used to determine the inhibitory effects of test extract on *in vitro* cell growth. The cancer cells from the mother culture were trypsinized (Fig. 1) using 0.25% trypsin/EDTA buffer. Further, trypsinized cells from the T-25 flask were seeded in each well of collagen coated 96-well flat-bottomed tissue culture plate with a density of 5×10^3 cells in the growth medium/well and incubated at 37 °C in 5% CO_2 to adhere. After 24 h of incubation, the supernatant was discarded and the cells were pretreated with growth medium followed by mixing with different concentrations of methanolic extract of Ulva fasciata (MEUF) (12.5, 25, 50, 100 and 200 μg/ml) in triplicates to achieve a final volume of 150 μl. The resultant mixture was then incubated for 48 h in CO_2 incubator (Acmas Technocracy Pvt. Ltd., ACM HF 160). The MEUF was prepared as 1.0 mg/ml concentration stock solutions in PBS. Culture medium and solvent are used as controls. Each well then received 20 μl of fresh MTT (5 mg/ml in PBS) followed by incubation for 4 h at 37 °C (Zhang et al., 2012). The supernatant growth medium was removed from the wells and replaced with 100 μl of DMSO to solubilize the colored formazan product. After 30 min of incubation, the absorbance (OD) of the culture plate was read at a wavelength of 492 nm on an ELISA reader (Anthos Biochrom 2020) and % cell survival and % cell inhibition were calculated.

2.5. Developing the experimental design

Various concentrations of MEUF and different cancer cell lines (HT-29, Hep-G2 and MCF-7) were inspected to identify the optimum cytotoxicity, leading to the following observations:

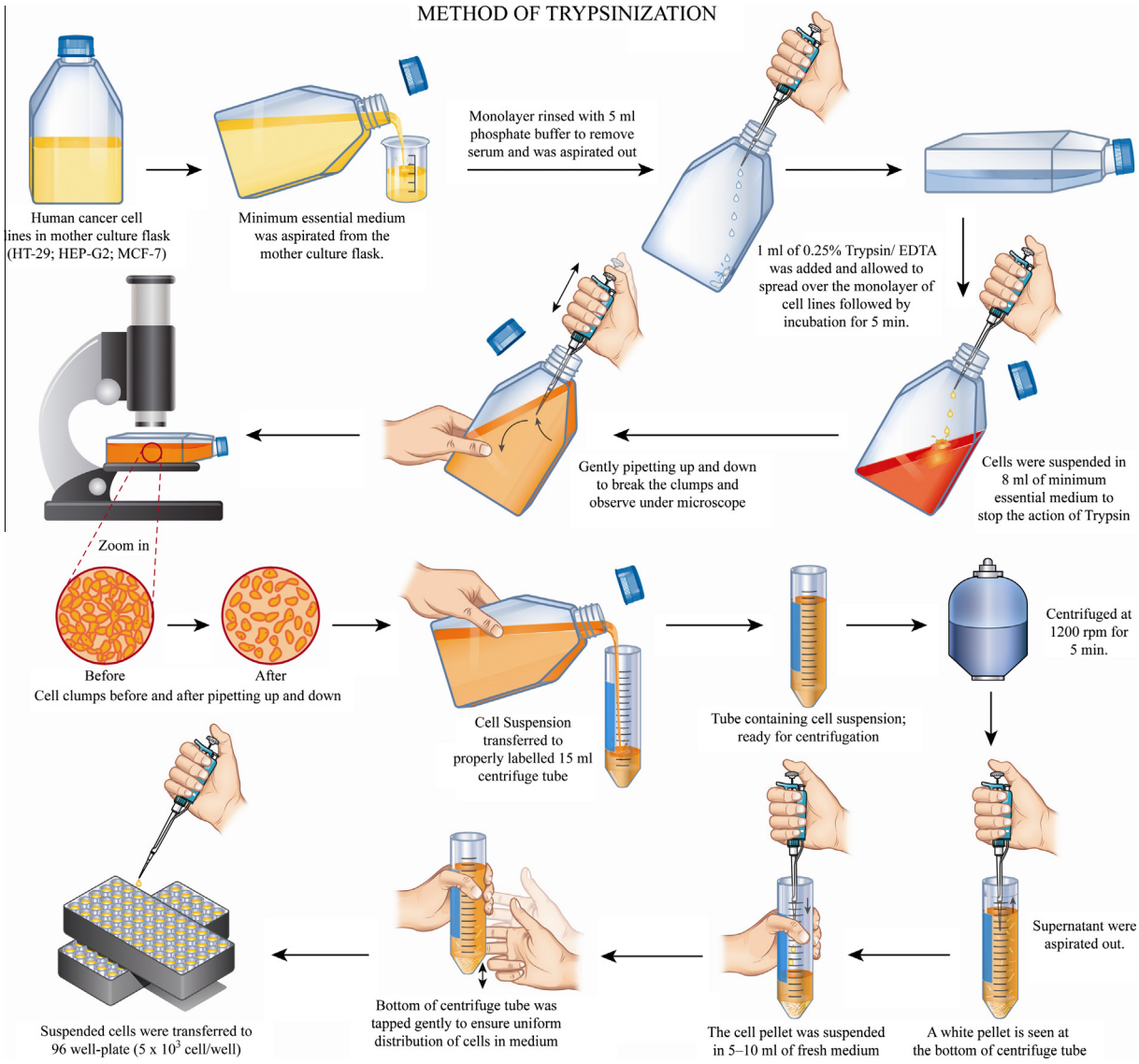

Figure 1 Method of trypsinization.

I. If the concentration increases, percent cell inhibition increases proportionately. If the concentration increased beyond 200 μg/ml, then higher superfluous toxicity was observed.
II. The percent cell inhibition depends on the nature of cell lines which provides the selectivity of MEUF.

Due to the aforementioned factors, a central composite design with the two independent variables at three levels was used to determine the response pattern and optimum combination of variables. The combined effect of concentration of MEUF (μg/ml) and cancer cell lines (HT-29, Hep-G2 and MCF-7) as independent variables on absorbance (OD), % cell survival and percent cell inhibition as dependent variables was investigated by the response surface methodology. The second order polynomial (regression) equation was used to represent the response surface and the value of coefficient was calculated using an Design expert 8.0.4 U.S.A software. Statistical comparison within variables was carried out by one-way ANOVA. A p-value less than 0.05 was considered to be statistically significant.

2.6. Conducting the experiments

According to central composite design, 13 experimental trials (MTT assays) were conducted for three different cancer cell lines each at three levels of concentrations (low, medium and high), and the translations of the coded levels are shown in Table 1. Cell inhibition was observed (Fig. 2) in comparison with the control using optical microscope equipped with camera.

3. Results

3.1. Data analysis

Analysis of variance (ANOVA) for the responses indicates that the assumed regression models were significant and

Table 1 Central composite design of two variables and the experimentally observed responses.

Run	X_1/concentration (μg/ml)	X_2/cancer cell lines	Y_1/absorbance at 492 nm	Y_2/% cell survival	Y_3/% cell inhibition
1	0 (125)	0 (Hep-G2)	0.595	71.65	28.35
2	1 (200)	−1 (HT-29)	0.339	58.4	41.6
3	0 (125)	−1 (HT-29)	0.396	68.88	31.12
4	0 (125)	0 (Hep-G2)	0.595	71.65	28.35
5	0 (125)	0 (Hep-G2)	0.595	71.65	28.35
6	0 (125)	1 (MCF-7)	0.472	71.92	28.08
7	1 (200)	0 (Hep-G2)	0.403	45.88	54.12
8	1 (200)	1 (MCF-7)	0.356	52.16	47.84
9	0 (125)	0 (Hep-G2)	0.595	71.65	28.35
10	−1 (50)	−1 (HT-29)	0.453	79.97	20.03
11	−1 (50)	0 (Hep-G2)	0.698	82.17	17.83
12	−1 (50)	1 (MCF-7)	0.516	77.8	22.2
13	0 (125)	0 (Hep-G2)	0.595	71.65	28.35

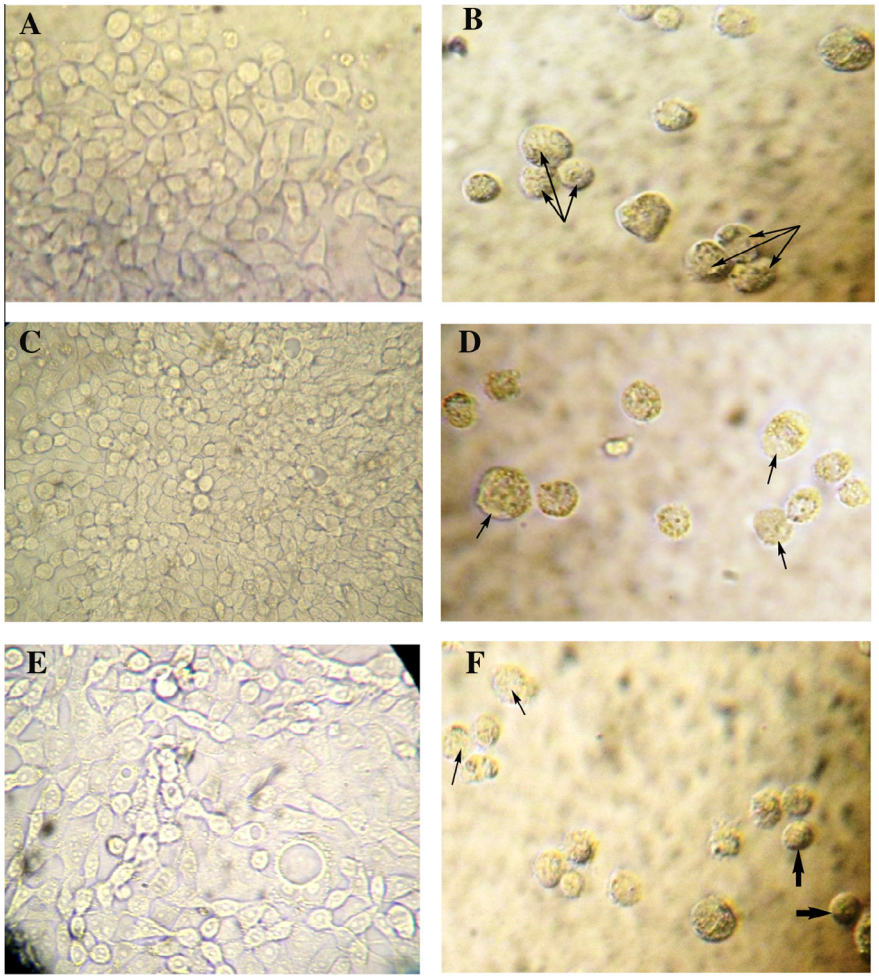

Figure 2 Photographs of cells under microscope; Fig. 2 (A, C, and E) and Fig. 2 (B, D and F) are photographs of controls and drug treated cells for HT-29, Hep-G2 and MCF-7 cell lines respectively. Fig. 2 (B and F) show few cells in clump and the cell walls are incompletely destroyed whereas Fig. 2(D) confirmed complete destruction of cell wall leading to the cell death.

valid for each of the responses ($p < 0.05$) shown in Table 2. The estimated coefficients for the experimental design are shown in Table 3. Response surface plots allow graphical visual observations that the regression analysis equations are significant. The regression Eqs. (1)–(3) were presented as response surface plots in Fig. 3 showing the combined effect of independent factors X_1 and X_2 on the responses Y_1, Y_2, and Y_3. From the polynomial regression equations, the influence of concentration on various cell lines was studied.

Table 2 Variance analysis for responses.

Source	Sum of squares	df	Mean square	*F* Value	*p*-value Prob > *F*	
Absorbance						
Regression	0.14	7	0.020	8.72	0.0149	Significant
Residual	0.012	5	2.326E-003			
Total	0.15	12				
% Cell survival						
Regression	1345.14	7	192.16	28426.45	< 0.0001	Significant
Residual	0.034	5	6.760E-003			
Total	1345.17	12				
% Cell inhibition						
Regression	1345.14	7	192.16	28426.45	< 0.0001	Significant
Residual	0.034	5	6.760E-003			
Cor. Total	1345.17	12				

df: degree of freedom; *F*: fisher ratio; *p*: probability.

Table 3 Estimated coefficients for the experimental design.

Factor	Coefficient Estimate	Standard Error	Confidence interval at 95% confidence		VIF
			Low	High	
Absorbance					
Intercept	0.60	0.022	0.54	0.65	
A-Concentration	−0.10	0.024	−0.17	−0.042	2.00
B-Cancer cell lines	0.027	0.024	−0.035	0.089	2.00
AB	−0.011	0.024	−0.073	0.050	1.00
A2	−0.041	0.018	−0.088	5.688E-003	1.02
B2	−0.100	0.018	−0.15	−0.053	1.02
R^2	0.9243				
Adj-R^2	0.8183				
% Cell survival					
Intercept	71.65	0.037	71.56	71.74	
A-Concentration	−12.83	0.041	−12.94	−12.72	2.00
B-Cancer cell lines	1.07	0.041	0.97	1.18	2.00
AB	−1.02	0.041	−1.12	−0.91	1.00
A2	−3.84	0.031	−3.93	−3.76	1.02
B2	−0.66	0.031	−0.74	−0.58	1.02
R^2	1.0000				
Adj-R^2	0.9999				
% Cell inhibition					
Intercept	28.35	0.037	28.26	28.44	
A-Concentration	12.83	0.041	12.72	12.94	2.00
B-Cancer cell lines	−1.07	0.041	−1.18	−0.97	2.00
AB	1.02	0.041	0.91	1.12	1.00
A2	3.84	0.031	3.76	3.93	1.02
B2	0.66	0.031	0.58	0.74	1.02
R^2	1.0000				
Adj-R^2	0.9999				

$$\text{Absorbance at 492nm} \rightarrow = +0.60 - 0.086A + 0.023B - 0.011AB - 0.041A^2 - 0.100B^2 \quad (1)$$

$$\%\text{Cell Survival} \rightarrow = +71.65 - 12.32A - 0.51B - 1.02AB - 3.84A^2 - 0.66B^2 \quad (2)$$

$$\%\text{Cell Inhibition} \rightarrow = +28.35 + 12.32A + 0.51B + 1.02AB + 3.84A^2 + 0.66B^2 \quad (3)$$

where, A is MEUF concentration (μg/ml) and B is human cancer cell lines.

The positive sign of the coefficient indicates that the dependent variable is greatly influenced and vice versa. From Eq. (1), it is found that the contribution of cancer cell line is more significant than the concentration of drug for absorbance at 492 nm. However, the interaction between the two has less influence on absorbance. In case of % cell survival (Eq. (2)), the influence of every individual variables and interaction between the variables showed negative significance effect. Among

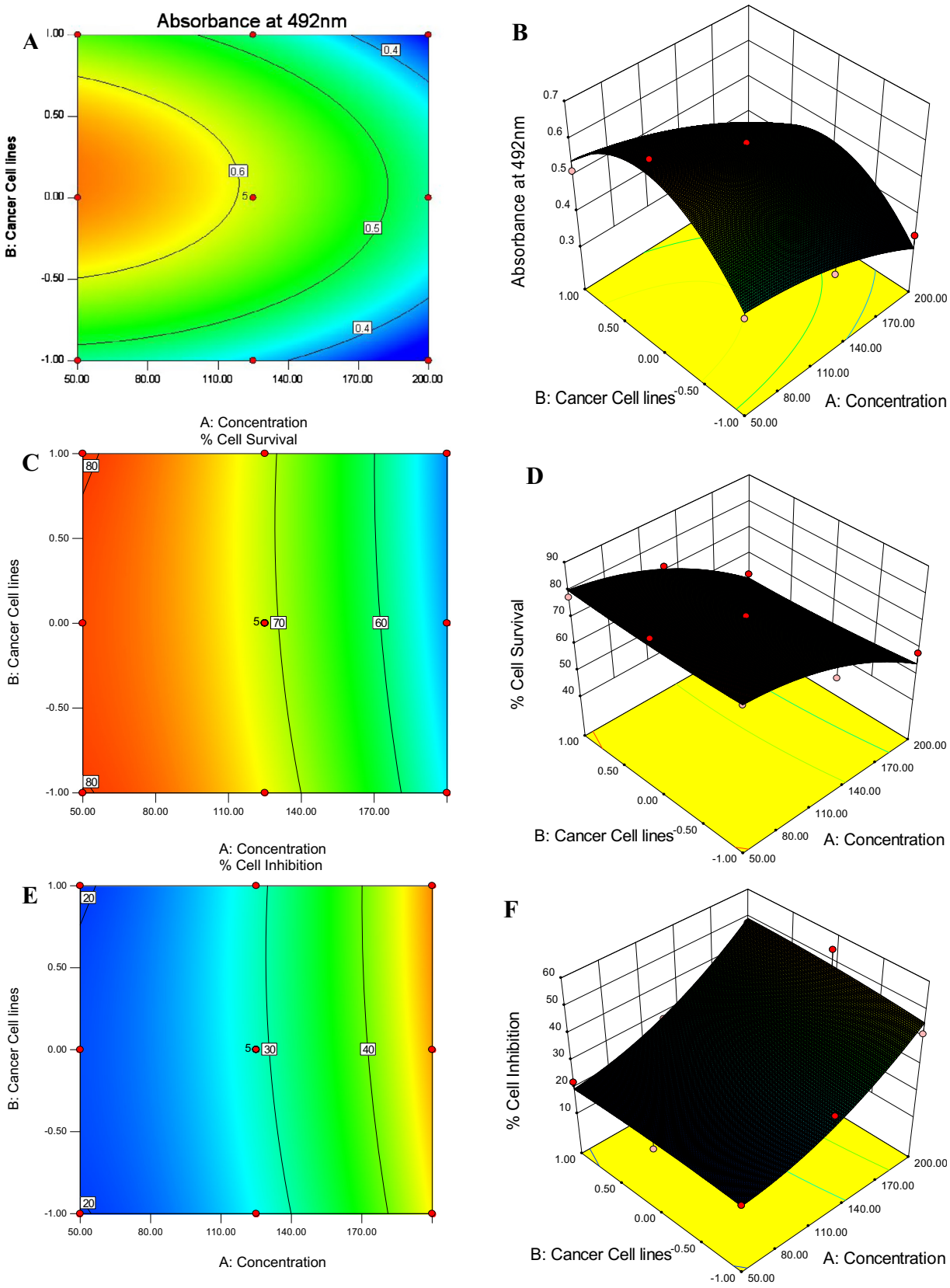

Figure 3 Response graphs and countour plots.

Table 4 Experimental design conditions and their results.

Sl No.	Concentration (μg/ml)	Cancer cell lines	Response variables	Observed response	Predicted response	Error	Mean percentage error
1	200	HT-29	Absorbance	0.341	0.37713	0.03613	1.20417
			% Cell survival	58.4	58.465	0.065	
			% Cell inhibition	41.6	41.535	0.065	
2	200	MCF-7	Absorbance	0.376	0.39413	0.01813	0.60417
			% Cell survival	52.16	52.225	0.065	
			% Cell inhibition	47.84	47.775	0.065	
3*	170*	Hep-G2*	Absorbance	0.451	0.46908	0.01808	0.60267*
			% Cell survival	57.88	57.1622	0.7178	
			% Cell inhibition	42.12	42.8378	0.7178	
4	168	Hep-G2	Absorbance	0.419	0.47759	0.05859	1.95283
			% Cell survival	57.18	57.729	0.549	
			% Cell inhibition	42.82	42.271	0.549	
5	166	MCF-7	Absorbance	0.486	0.46222	0.023776	0.792533
			% Cell survival	57.15	57.3269	0.1769	
			% Cell inhibition	42.85	42.6731	0.1769	
6	156	MCF-7	Absorbance	0.456	0.47682	0.02082	0.6941
			% Cell survival	58.66	58.4812	0.1788	
			% Cell inhibition	41.34	41.5188	0.1788	
7	180	Hep-G2	Absorbance	0.403	0.46627	0.06327	2.1089
			% Cell survival	57.88	56.9898	0.8902	
			% Cell inhibition	42.12	43.0102	0.8902	
8	188	HT-29	Absorbance	0.393	0.42957	0.03657	1.2189
			% Cell survival	58.4	57.9648	0.4352	
			% Cell inhibition	41.6	42.0352	0.4352	

* Showing the best combination of concentration and cancer cell line for optimum cytotoxicity with lowest mean percent error.

all, highest negative influence was observed by the concentration of drug. Whereas, % Cell Inhibition (Eq. (3)) is greatly influenced by individual concentration or cell line, or their combined interaction and the role of concentration is predominant.

3.2. Validation of model and optimization

To validate the regression analysis equation, 8 check point solutions were chosen for experimentation. The predicted and observed values of the dependent variables were calculated (Table. 4) and found a close agreement between the values. The optimum cytotoxicity was found when Hep-G2 was treated with 170 μg/ml of MEUF with lowest mean percent error. At this optimized condition the study showed absorbance of 0.451% and 0.46908%; cell survival of 57.88 and 57.1622; % cell inhibition of 42.12 and 42.8378 for observed and predicted values respectively.

4. Discussion

Natural products in many parts of the world are used for the treatment and prevention of cancer. Though these products many a case are not more potent than conventional synthetic drugs like cisplatin, fluorouracil, and cyclophosphamide but are less toxic (Pan et al., 2012). So many bioactive compounds have been isolated from numerous marine algae to treat dreaded disease like cancer and HIV (Jha and Zi-rong,

2004). Copious poly saccharides from natural sources reported to have been promising antineoplastic activity (Wu et al., 2012; Zhang et al., 2004, 2007; Cho et al., 2010). Many other species of Ulva have proven anticancer activity (Kaeffer et al., 1999) because of the presence of sulfated polysaccharide. Keeping the above statement in mind we have designed to study the cytotoxic potential of *Ulva fasciata* Delile which is unreported yet. Our present study reveals that the relative cell survival decreased in a dose-dependent manner in all the cancer cell lines, but the percentage of inhibition is more in case of Hep-G2 cells as compared to HT-29 and MCF-7.

5. Conclusion

Response surface methodology was applied using central-composite experimental design to obtain optimum combined effect of concentration and cancer cells with highest cytotoxicity. The aforementioned study substantiated a higher cytotoxic potency of MEUF on Hep-G2 as compared to HT-29 and MCF-7 cells. Though the cytotoxicity is not very much significant, further studies are in progress to enhance the potency of the MEUF in combination with other species of algal extract from marine source and plant extracts from terrestrial source, as polyherbal extracts (PHE). In addition to this further scope is there to establish these extracts as polyherbal formulations having potent anti-cancer activity.

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